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Orally Active Carbamate Prodrugs of the Selective Dopamine Agonist N-0437: In-vivo Activities in the 6-OHDA Turning Model and In-vitro Activities

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Abstract—The in-vivo activities of eight carbamate prodrugs of the D_2-agonist N-0437 were determined by examining the effects of the prodrugs, after their oral administration in rats with unilateral 6-OHDA lesions of the striatum. The resulting contralateral turning was used as an index of the activity of the compounds. A comparison of the area under the curve of the time-effect curves of the prodrugs, revealed a significantly improved duration of action compared with N-0437 during the period 11–15 h after administration, for the propylcarbamate and the dimethoxyphenylcarbamate derivatives. The 2,4-dimethylphenylcarbamate showed a significantly enhanced turning behaviour over the whole 15 h time interval in comparison with N-0437. Three of the nine carbamates were virtually unhydrolysed in rat serum at 37°C, while the other test compounds were hydrolysed relatively slowly, with $t_{\frac{1}{2}}$ values ranging from 1·5–6 h. The test compounds differed greatly in partition coefficients, which were estimated by RP-HPLC (1–12 times more lipophilic than N-0437). The potential cholinesterase inhibiting properties of the carbamate prodrugs were assessed by a simple in-vitro incubation assay, which showed that only two carbamates were very weak cholinesterase inhibitors.

The use of drug derivatives which are converted to the active compound within the body by enzymatic or chemical processes has become a useful approach to improve drug delivery (Sinkula & Yalkowsky 1975; Bundgaard 1984, 1985).

N-0437 (5-hydroxy-2-(*N*-n-propyl-*N*-2-ethylthienyl-amino)tetralin) is a very potent and selective D_2-agonist (Van der Weide et al 1987) and was shown to be active in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced parkinsonian model in the marmoset (Löschmann et al 1989). However, N-0437 undergoes considerable inactivation by glucuronidation in gut and liver in rat and monkey (Gerding et al 1990).

It has been suggested that in order to control fluctuations in motor performance and involuntary movements in Parkinson's disease, an ideal dopamine agonist should be able to sustain its occupation of dopamine receptors for a long period (Löschmann et al 1989). Thus a prodrug of N-0437 with a long duration of action would be of interest for the treatment of Parkinson's disease.

In an attempt to solve the problem of extensive first-pass metabolism several ester and carbamate prodrugs were synthesized of which the ester prodrugs have been described elsewhere (den Daas et al 1990). Carbamate prodrugs were also investigated because many of these compounds are known to be reversible cholinesterase inhibitors. This property may minimize first-pass hydrolysis of the prodrug by esterases present in the gut wall, blood and liver (Tunek & Svensson 1988).

The testing of prodrugs in behavioural models is frequently used to investigate their in-vivo potencies (Cooper et al 1987; Broekkamp et al 1988; den Daas et al 1990). Eight carbamates of N-0437 (Fig. 1) were therefore tested in a behavioural model which reflects postsynaptic activity, i.e. contralateral turning in the rat after a 6-hydroxydopamine (6-OHDA) lesion.

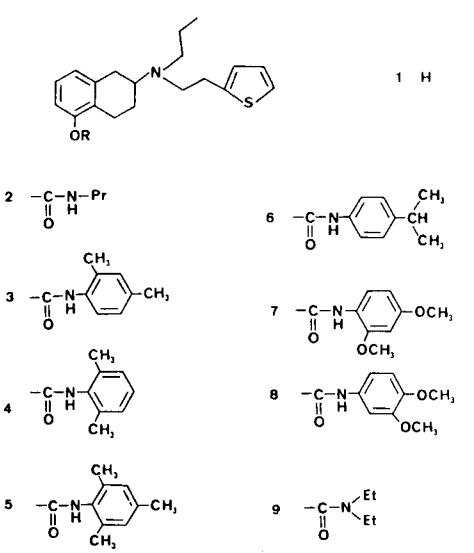

FIG. 1. Structures of N-0437 and eight carbamate prodrugs.

In-vitro parameters which can be expected to be relevant for the in-vivo activity of the prodrugs, hydrolysis rates and lipophilicity, were determined for all test compounds. Hydrolysis rates were determined in rat serum and lipophilicities were determined by reversed phase HPLC. In addition, the potential cholinesterase inhibiting properties of the carbamates were assessed by measuring choline after incubation of the compounds with acetylcholine and cholinesterase.

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Materials and Methods

Synthesis

N-0437 was synthesized according to the method of Horn et al (1985). The one-step reaction of the appropriate carbamoyl chloride derivatives with N-0437 to form the carbamates was carried out as described by Thorberg et al (1987). The yield of the reactions of the carbamate compounds (2–9), isolated as their HCl salts, varied from 56–76%.

Animal experiments

N-0437 and the carbamate compounds were dissolved in a solution of alcohol, water and polyethyleneglycol (3:3:4) for oral administration, or in dimethylsulphoxide for in-vitro tests. 6-OHDA (Sigma, St Louis, USA) was dissolved in a solution of 0·9% sodium chloride containing ascorbic acid (3 mg mL^{-1}). Apomorphine HCl (Brocacef, Maarssen, The Netherlands) was dissolved in water containing ascorbic acid (3 mg mL^{-1}). All solutions were freshly prepared before use.

Male albino Wistar rats, 200–300 g, (CDL, Groningen) were housed in plastic cages (55 × 35 × 20 cm) in groups of five and had free access to food and water.

Surgery and turning behaviour

Rats were anaesthetized with chloral hydrate (400 mg kg^{-1} i.p.) and placed in a stereotaxic instrument (Kopf). After removing skin and tissue a small hole was drilled in the skull and 6-OHDA (8 μg dissolved in 1 μL 0·9% NaCl containing 3 mg mL^{-1} ascorbic acid) was injected in the left medial forebrain bundle at coordinates A 4·8, L 2·2 and V 2·4 (König & Klippel 1963) to destroy the dopaminergic input to the striatum. Successfully denervated animals were selected one week after surgery by testing the response to 2 μmol kg^{-1} apomorphine for their frequency of turning.

Rotational behaviour was automatically recorded in rotometers according to the method of Ungerstedt & Arbuthnott (1970). Only rats showing more than 100 contralateral turns per 30 min were selected for the experiments. Animals which did not show sufficient turning behaviour were tested one week after the first test. When again no sufficient turning behaviour was apparent the rats were excluded. Approximately 80% of the operated rats were selected for the experiments and were used 4 times for a 20 h experiment with a resting interval of one week.

Before the experiments the rats were fasted overnight. After oral administration of the test compound (2 mL kg^{-1}) the rats were placed in the rotometer. Registration of turning behaviour started 10 min after injection. The time of each contralateral and ipsilateral turn of a maximum of 6 rats was registered on a microcomputer. For the comparison between N-0437 and the prodrugs all doses were equimolar, i.e. 50 μmol kg^{-1}.

After the experiments the rats were decapitated and the striata were dissected for determination of striatal dopamine. All rats which performed well after apomorphine injection showed more than 95% striatal dopamine depletion compared with the non-lesioned side.

In-vitro hydrolysis

The hydrolysis profiles of the carbamate prodrugs were determined in rat serum which was collected from rats by puncture of the aorta abdominalis after ether anaesthesia. The serum was kept frozen until the start of the experiments. Hydrolysis of the prodrugs was followed in 3 mL serum at 37°C with an initial prodrug concentration of 35 μM. At various incubation times, a 200 μL aliquot of serum was taken and added to 2 mL of acetonitrile to precipitate proteins. The extraction procedure and the assay for N-0437 by HPLC and electrochemical detection were as previously described (den Daas et al 1989).

Two prodrugs, 2,4-dimethylphenylcarbamate (3) and 2,5-dimethylphenylcarbamate (4), were unstable during the extraction procedure. An extraction was therefore omitted for these compounds; instead 1 mL 0·4 M perchloric acid was added to the samples and the supernatant was directly injected onto the HPLC column. To validate this method several other prodrugs were tested this way and both methods gave comparable results.

All carbamate prodrugs proved to be stable in the two solvents used for the in-vitro and in-vivo experiments. After dissolution and storage at ambient temperature only 5% of any carbamate prodrug was hydrolysed after 3 days.

Lipophilicity

Since we had previously determined the octanol-buffer (pH 7·4) partition coefficient of N-0437 by the shake-flask method (log P = 3·20), partition coefficients of the test compounds could be assessed by comparison of their capacity factors with N-0437 on a reversed phase column.

The retention times of N-0437 and the prodrugs were determined on an HPLC system with UV detection (HPLC-UV, Waters Associates, Milford, MA, USA) at 234 nm. Separation was performed, after an injection of 1·0 mM of the compound, on a 250 × 4·6 mm I.D. Chrompack (Middelburg, The Netherlands) ChromSpher C8 reversed-phase column with 5 μm particle size. The mobile phase (pH 4·3) consisted of a solution of 65 mM citric acid and 70 mM dibasic sodium phosphate, 35% (v/v) 2-propanol, 0·3% (v/v) triethylamine and 100 mg L^{-1} Na_2EDTA. The mobile phase was filtered through a 0·45 μm filter (Millipore) before use.

Cholinesterase inhibition

To investigate the cholinesterase inhibiting properties of the various compounds an in-vitro test system was developed.

Acetylcholine (0·5 mL, 0·1 mM) (Sigma, St Louis, USA) and 0·1 mL (10 μM) of the carbamate compound were diluted in 0·4 mL 0·2 M phosphate buffer (pH 8·0) and incubated at 37°C. To start hydrolysis, 10 μL 0·10 units acetylcholinesterase (Sigma, St Louis, USA) was added and 10 min later the reaction was stopped by heating the test tube in boiling water for 30 s. After addition of 9 mL phosphate buffer the concentrations of acetylcholine and choline were measured by HPLC-ECD according to the method of Damsma et al (1985).

Blank (no esterase added) and control (no test compound added) incubations were included in each experiment. Inhibition of the cholinesterase activity was expressed as percentage inhibition of the control values (no inhibitor present: 100% hydrolysis).

To check the validity of this assay, various experiments were carried out with neostigmine HBr (Centra Chemie, Etten Leur, The Netherlands), which is a potent cholinesterase inhibiting compound.

Statistics

The data from the behavioural experiments were analysed by use of a spreadsheet program (123-Lotus), which calculated the time-activity profile of the drug and the area under the curve (AUC) of 4 time intervals as a measure of the activity of the compound. Statistical analysis was performed with the Dunnett's *t*-test of a control versus several treatments (Grimm 1973). The hydrolysis profiles were calculated by non-linear curve fitting of the data points with the least squares method using the equation $y = bx/(a-x)$.

Results

The activity of N-0437 and the carbamate prodrugs in the 6-OHDA turning model

The time response profiles of N-0437 (1) and the carbamate prodrugs (2–9) showed a diverse pattern (Fig. 2). The time response profile of N-0437 is typical for a lipophilic drug with a high first-pass metabolism. Almost immediately after p.o. administration there is a period of fast contralateral turning which reaches its maximum in the first hour and then decreases rapidly until at 5 h the rats are turning at approximately 175 turns h^{-1}. After 5 h the decline is smaller until, at 7 h, it reaches a point at which turning behaviour is not detectable compared with vehicle administration.

The propylcarbamate (2) shows a similar pattern to that of N-0437. The potency of this prodrug is less in the first 5 h after administration, although not significantly so (Fig. 3), but it is significantly more potent in the third time interval from 11–15 h. No difference in potency is observed between the propylcarbamate (2) and N-0437, in the period from 1–15 h. The diethylcarbamate (9) shows no pharmacological effect at all after oral administration.

The 2,4-dimethylphenylcarbamate (3) shows the same profile in the first 5 h (Fig. 2) and the same potency (Fig. 3) as the propylcarbamate (2) but after 5 h the turning activity remains at a high level until the end of the experiment at 20 h after administration. A significant improvement in potency occurs in the time intervals 6–10, 11–15 and 1–15 h, although there are large fluctuations in the time response profile of the compound.

No significant increase in potency compared with N-0437 was found after oral administration for the methylphenylcarbamate compounds (4, 6). The 2,4,6-trimethylphenylcarbamate (5) shows no activity at all.

The profiles of the two methoxyphenylcarbamate analogues (7, 8) show a different time-response activity (Fig. 2). Very little effect can be seen immediately after administration. The turning activity increases slowly after 1 h and stays stable until 14 h after administration. The potency of these two compounds is significantly higher with respect to N-0437 in the time interval from 10–15 h (Fig. 3).

Hydrolysis of prodrugs

All carbamate prodrugs showed a relatively slow hydrolysis pattern (Fig. 4) especially when compared with the ester prodrugs of N-0437 (den Daas et al 1990). The compound with the highest hydrolysis rate was the 2,5-dimethylphenylcarbamate (4) with a half-life ($t_{\frac{1}{2}}$) of 100 min. The total amount of prodrug conversion, calculated as the area under the curve (AUC), over the time interval 0–180 min after incubation, is estimated to be up to 45% of the theoretical amount of N-0437 (Table 1).

Lipophilicity

HPLC can be used as a rapid and reproducible method to

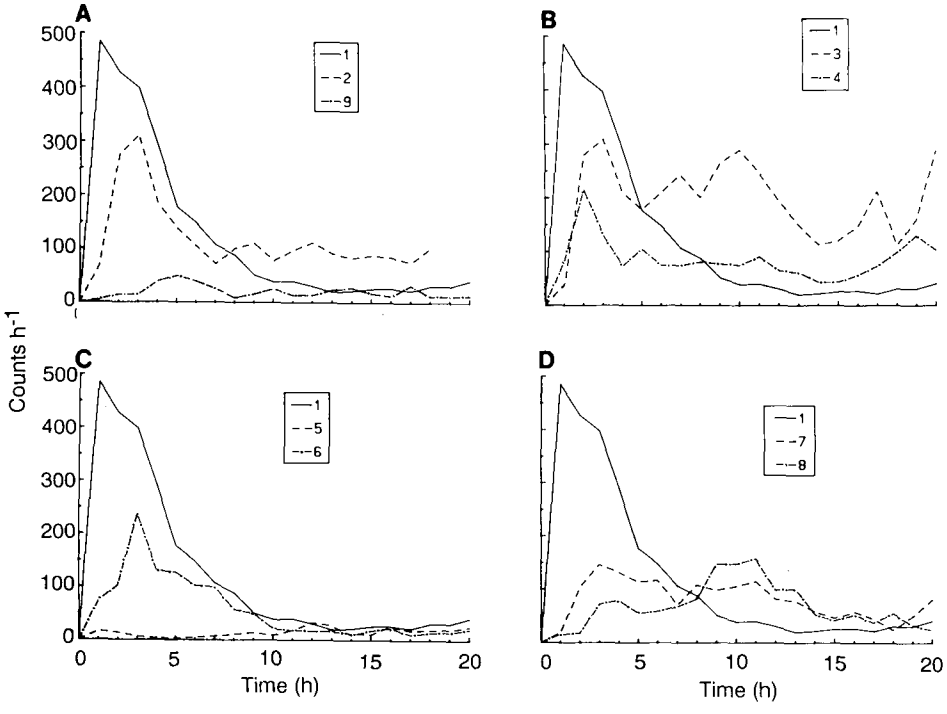

FIG. 2. Effect of p.o. administration (50 μmol kg^{-1}) of N-0437 (1) and its prodrugs (2–9) on contralateral turning behaviour (n = 9).

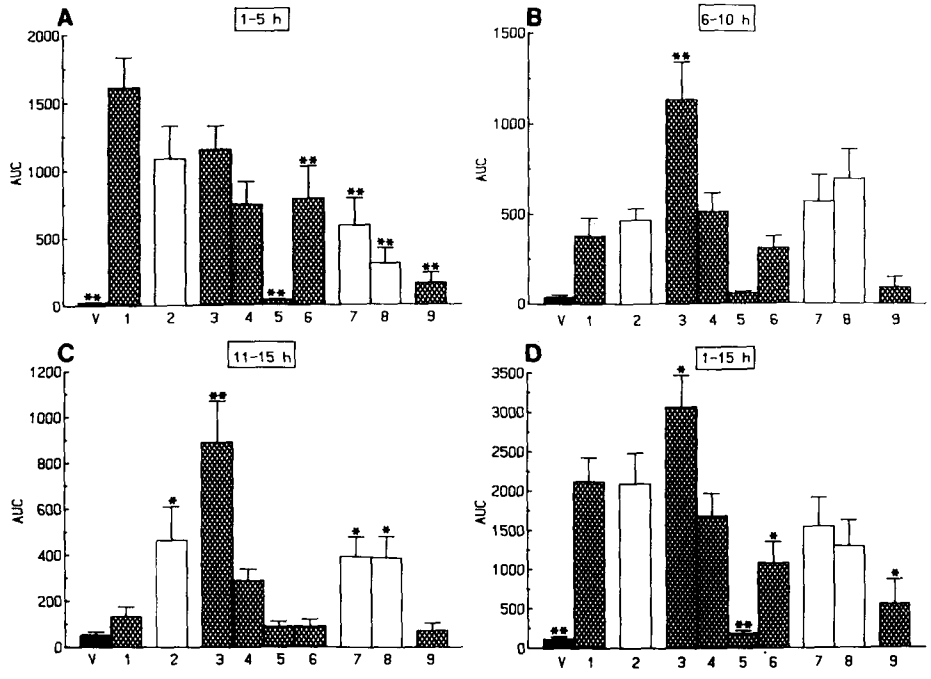

FIG. 3. The AUC from the time-response profile of N-0437 (1), vehicle (V) and prodrugs (2–9) after p.o. administration (50 μmol kg^{-1}) calculated for the time intervals 1–5, 6–10, 11–15 and 1–18 h (error bars are s.e.m., n = 9, $*P < 0.05$, $**P < 0.01$) vs N-0437, Dunnett's test).

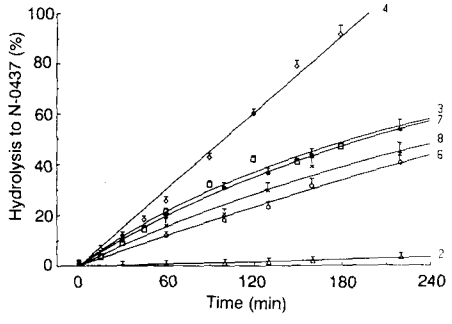

FIG. 4. The in-vitro hydrolysis in rat serum of the prodrugs of N-0437. Error bars are s.e.m.; n = 4.

obtain lipophilicity values which correlate well with partition coefficient (Dearden & Bresnen 1988). In order to be able to compare lipophilicities, all retention times were measured with the same mobile phase and flow rate and were expressed relative to N-0437 (k′ = 1·0) (Table 1). These capacity factors can be transformed to log P values since the experimental log P (octanol/water) of N-0437 has been determined to be 3·2. The experimental lipophilicities so obtained are in good agreement with the theoretical, calculated partition coefficients (Martin 1978), based on the experimental log P of N-0437.

Cholinesterase inhibition

The conversion of actylcholine to choline in the presence of cholinesterase is not inhibited by N-0437 (1) and most of the carbamate analogues, e.g. 2, 4, 5, 6, 7 and 9. Weak inhibition was observed only after incubation with the 2,4-dimethylphenylcarbamate (3) and the 3,4-dimethylphenylcarbamate (8) with inhibition of choline formation to approximately 90% of control values.

For comparison the effect of the potent inhibitor neostigmine was measured. After incubation of 0, 0·01, 0·1 and

Table 1. In-vitro parameters and lipophilicity values of N-0437 and eight carbamate prodrugs.

Compound	Hydrolysis $t_{\frac{1}{2}}$ (min)	Hydrolysis AUC (%)	k′ (rel. to N-0437)	log P	AChE inhibition (remaining ACh)
1	—	—	1·00	3·2	0·97 ± 0·03
2	≫1400	1	1·35	3·3	0·95 ± 0·04
3	188	28	2·09	3·5	0·90 ± 0·01
4	100	45	1·83	3·5	0·96 ± 0·03
5	—	—	12·25	4·3	0·93 ± 0·05
6	314	18	4·14	3·8	1·00 ± 0·06
7	191	27	1·71	3·4	0·96 ± 0·04
8	369	22	1·27	3·3	0·88 ± 0·01
9	—	—	1·81	3·5	0·96 ± 0·04
Control	nd	nd	nd	nd	1·00 ± 0·01
Neostigmine	nd	nd	nd	nd	0·22 ± 0·01

nd, not determined.

1·0 μM of neostigmine for 10 min, the reaction from acetylcholine to choline was inhibited to respectively 100, 94, 74 and 22% of control values. Thus neostigmine is approximately 100 times more potent than the carbamates 3 and 8 in inhibiting the conversion of acetylcholine to choline.

Discussion

No clear and simple relation could be found between in-vitro hydrolysis rates, lipophilicity, cholinesterase inhibiting activity and in-vivo activities. Several authors (Notari 1985; Hussain et al 1987) found no relation between prodrug bioavailability and in-vitro conversion and although those studies examined mainly pharmacokinetic parameters such as plasma concentrations, care has to be taken in comparing in-vitro experiments with a pharmacological effect. Nevertheless in-vitro prodrug hydrolysis can provide a measure of relative susceptibility of analogues to enzymatic attack.

All carbamate prodrugs investigated in this study show a slow hydrolysis rate compared with the aliphatic ester prodrugs of N-0437 described in a previous study (den Daas et al 1990). This slow hydrolysis is probably due to esterase inhibition by the carbamate structure, as found in the study of carbamate prodrugs of terbutaline (Tunek & Svensson 1988).

The high in-vivo activity of the 2,4-dimethylphenylcarbamate (3) could be the result of an ideal hydrolysis profile ($t_{\frac{1}{2}} = 188$ min) combined with appropriate lipophilicity (log $P = 3{\cdot}5$) and weak esterase inhibiting properties, as found in the in-vitro assay. The increase in lipophilicity due to an aromatic carbamate group could cause an accumulation of the prodrug in the fatty tissues of the organism after administration, with a resulting slow release into the blood circulation upon hydrolysis of the prodrug. This compartmental accumulation has also been described for other compounds including oestradiol derivatives (Giannina & Meli 1969; Gardi et al 1973).

The N-0437-like in-vivo activity of the propylcarbamate (2) in relation to the extremely slow in-vitro hydrolysis of this compound is difficult to explain, but it is possible that this can be due to other and faster enzymatic cleavage systems in, for example, liver or brain tissue.

A possible disadvantage of using aromatic carbamate prodrugs is their potential central cholinesterase inhibiting activity (Kolbezen et al 1954), which can cause convulsions, and in certain cases damage to the liver due to aniline hydrolysis products (Ioannou et al 1988). No convulsions have been seen with the prodrugs investigated in this study which is in agreement with the absence of anti-cholinesterase activity of these carbamate analogues as established in the in-vitro assay.

Because of these potential problems the practical use of this group of compounds may be limited. However, this study illustrates the methodology of a fast and systematic screening of these kinds of compounds in a functional model, which may be used to find less toxic prodrug derivatives with the desired activity profile.

In a comparative study on carbamate derivatives of the dopamine agonist 3-PPP (3-(hydroxyphenyl)-*N*-propylpiperidine) (Thorberg et al 1987) the greatest pharmacological effects were measured with the propyl carbamate and the 4-isopropylphenylcarbamate analogues. Although only plasma levels of 3-PPP were measured it is obvious that the structure activity relations of the two sets of drugs are different. It would thus appear that it is difficult to use information on structure-activity-relationships from one series of dopaminergic prodrugs for the design of another series.

In spite of the possible toxic effects arising from the production of 2,4-dimethylaniline, the 2,4-dimethylphenylcarbamate (3) is the most promising compound of the examined carbamate analogues, which is probably due to an optimal balance between hydrolysis, cholinesterase inhibiting activity and lipophilicity. A non-toxic phenylcarbamate with a similar pharmacokinetic profile could, in theory, be useful for the treatment of Parkinson's disease.

Acknowledgement

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